Physical Characterization and Cellular Testing of Polymeric Micelle Formulations for Intravenous Delivery of Hydrophobic Anticancer Drugs

by

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Abstract

Colorectal cancer ranks amongst the top three types of cancer diagnosed in the United States and is the second most common cause of cancer death. There are several drugs approved for the treatment of colon cancer. However, one of the more commonly used treatments, immune checkpoint inhibitors, are only effective in around 15% of patients diagnosed with metastatic colon cancer. Furthermore, many anti-cancer drugs specific to colon cancer are hydrophobic and thus have poor water solubility. Therefore, there is a need for a delivery formulation that is water soluble so that the drug can be introduced to the blood stream. With these requirements in mind, the overarching goal of this project is to develop a delivery system using micelles that are water soluble, and that can hold hydrophobic anti-cancer drugs. To achieve this goal, there are a few characteristics of the micelles that need to be ascertained to determine their suitability in tumor treatment. These include critical micelle concentration (CMC), encapsulation efficiency, size and

cytotoxicity. These characteristics denote the concentration at which micelles form, how much drug they can hold, their diameter, and whether they are toxic to cells.

Previous research done for this project investigated a new hydrophobic drug with which to treat colon cancer, as well as a micelle formulation to facilitate the delivery of this drug. The new drug is 15-deoxy, $\Delta^{12,14}$-PMJ_2 and was originally investigated for use against melanoma cancer but has shown promising results for treating colon cancer. The first micelle formulation used was a 1:5 ratio of DSPE:TPGS, where DSPE is a polyethylene glycol conjugated phosphatidylethanolamine and TPGS is a polyethylene glycol derivative of α-tocopherol (vitamin E). These micelles exhibited promising characteristics such as a low CMC, a high encapsulation efficiency when loaded with drug, and an appropriate size for nanoparticles used in cancer treatment (between ≈ 10-100 nm). However, it was discovered that this formulation was cytotoxic towards cancer cells, so the project pivoted to investigate pluronic F127 micelles as an alternative delivery system. Pluronic is the trade name for triblock copolymers composed of a central hydrophobic chain of polypropylene oxide (PPO) which is between two hydrophilic chains of polyethylene oxide (PEO); the F127 designation denotes the structure $PEO_{100}PPO_{65}PEO_{100}$. These micelles were also found to have a low CMC, although not as low as DSPE:TPGS, a high encapsulation efficiency, and an appropriate size for tumor treatment. In addition to these findings, the micelles also showed little to no cytotoxicity towards cancer cells. Thus, pluronic F127 micelles are now the primary candidate for the delivery of PMJ for future research projects.

Physical Characterization and Cellular Testing of Polymeric Micelle Formulations for Intravenous Delivery of Hydrophobic Anticancer Drugs

A Thesis

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In Partial Fulfillment of the Requirements for the Degree

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by

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List of Abbreviations

ICI: Immune Checkpoint Inhibitor

MSI-H: Microsatellite Instability High

MSI-L: Microsatellite Instability Low

dMMR: Mismatch Repair Defficient

PMJ: 15-deoxy, $D^{12,14}$,-prostaglandin J^2-ethanolamide

DSPE: 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine polyethyleneglycol-2000

TPGS: D-α-tocopheryl polyethylene glycol 1000 succinate

CMC: Critical Micelle Concentration

EE: Encapsulation Efficiency

ASL: Asulacrine

PEO: Polyethylene oxide

PPO: Polypropylene oxide

NMR: Nuclear Magnetic Resonance

CD: Circular Dichroism

PGJ: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2

TBTU: O-(benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate

DIEA: Diisopropylethylamine

TLC: Thin Layer Chromatography

HPLC: High Performance Liquid Chromatography

TEM: Transmission Electron Microscopy

DLS: Dynamic Light Scattering

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium

PBS: Phosphate Buffered Saline

PDI: Polydispersity Index

DI: Deionize

UPR: Unfolded Protein Response

ER: Endoplasmic Reticulum

ACF: Autocorrelation Function

BCEC: Brain capillary endothelial cells

CCK-8: Cell Counting Kit -8

WST-8: 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium

Chapter 1: Introduction and Background

1.1 Cancer

Much of the US population understands cancer to be a seemingly unstoppable disease whose diagnosis is a death sentence. However, reality is not so bleak as many believe; decades of research and countless years of scientific observation have culminated in a previously unheard-of understanding of cancer. For years, it was understood that cancer causes cells to proliferate uncontrollably, but the how was unclear. Over time it was discovered that two things had to happen for cancer to arise in a patient: first, a mutation that leads to continual growth signals in a cell. Second, a mechanism that turns off that growth signal must fail. Moreover, cancer doesn't just erupt throughout the body all at once. Clinicians describe the extent of a cancer diagnosis using a classification system which uses numbers to denote the stages of cancer progression. It occurs in stages ranging from zero to four, beginning first in a specific location as abnormal, noncancerous cells (stage zero). These cells can then progress to form a small, cancerous tumor that qualifies as stage one. In stages two and three, the tumor grows larger and deeper into surrounding tissue, with stage three being characterized by infiltration into nearby tissues such as the lymph nodes. Cancers can then enter their terminal stage, otherwise known as stage four, at which point the cancer cells have traveled throughout the body and formed new tumors in other tissues and organs.[1] This movement of cancerous cells throughout the body is known as metastasis.

Further experience with treating and researching cancer led to the conclusion that not all cancers are the same and that they are specific to cell types and genotypes. This is usually based

on where in the body the cancer has developed, and more specifically what kind of tissue the cancerous cells have infiltrated/affected. For example, cancer found in the breast is classified as breast cancer, cancer found in the lungs is lung cancer, and so on and so forth. For this project, colon cancer is of particular interest.

1.1.1 Colon Cancer

Colorectal cancer ranks amongst the top three types of cancer diagnosed in the United States and is the second most common cause of cancer death.[2] Colon cancer develops in stages, starting out as polyps (or adenomas) formed from mucosa exhibiting dysplasia (formation of abnormal cells). If these polyps are detected early, usually through screening via a colonoscopy, they can be removed and reduce the chances of developing cancer. However, if the polyps are not removed, they can then grow to become cancerous, or adenocarcinomas.[3] Cancerous cells from these adenocarcinomas can then spread throughout the body, becoming metastatic. Once colon cancer has reached the metastatic stage, the rate of survival is greatly diminished. Sadly, upon diagnosis, around 20% of people have reached the metastatic stage.[4]

Once diagnosed with colon cancer, one of the first tests done is for the microsatellite instability (MSI) biomarker. MSI refers to frameshift mutations in microsatellite DNA caused by the disruption of the DNA repair mechanism, mismatch repair (MMR). This mechanism ensures that the bases in DNA were incorporated correctly during the cell cycle. MSI screening allows for the deduction of important characteristics and prognostics regarding a patient's colon cancer diagnosis, and there are two statuses associated with this biomarker: microsatellite instability low (MSI-L) and microsatellite instability high (MSI-H). MSI-L indicates that less than 30% of specific microsatellite biomarkers being screened contain mutations or are unstable. MSI-H indicates that greater than 30% of the biomarkers are unstable.[5]

1.2 Chemotherapy

Chemotherapy is a form of cancer treatment that uses anti-cancer drugs, often in combination with other forms of treatment such as radiation, to inhibit or kill cancerous cells. It can also be used to shrink tumors so that they can be removed surgically.[6] It is an effective form of cancer treatment for many, but chemotherapy also comes with a myriad of potentially debilitating side effects. There is also the issue of patients developing drug resistance during treatment, thus necessitating alternative therapeutics with different mechanisms of action, often used in combination with each other to prevent cancer cells from mutating during treatment. A commonly used chemotherapeutic that is used in drug combinations to treat colon cancer is irinotecan HCl (brand name Camptosar, see Figure 1 for structure). Irinotecan HCl is a DNA topoisomerase I inhibitor that essentially breaks the DNA in cancer cells to kill them.[7] If combinations including irinotecan HCl are found to be ineffective, then it is common practice to switch to immunotherapy. Immunotherapy (classified as immune checkpoint inhibitors or ICIs) works by stimulating the immune system to kill cancer cells, which is very effective except when it comes to colon cancer. ICIs are only effective in about 5% of patients, hence the need for new therapeutics for colon cancer treatment.[4]

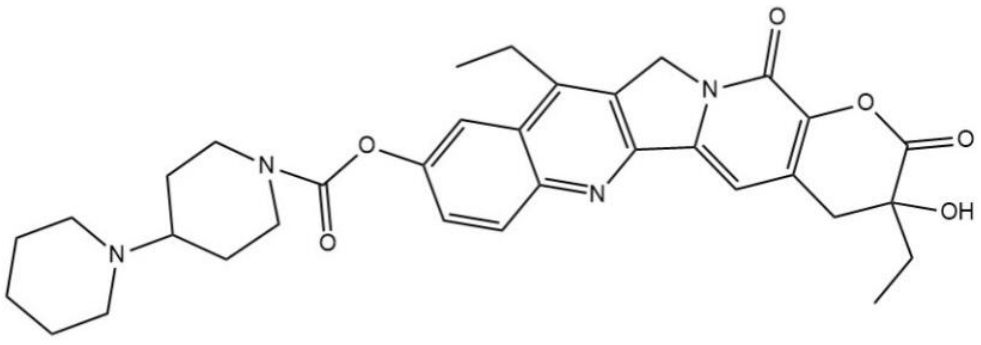

Figure 1 Structure of irinotecan HCl (Camptosar).

1.2.1 Immune Checkpoint Inhibitors

ICIs work with the immune system to target and eliminate cancer cells in patients. There are three types of ICIs in use today: 1) Programmed Cell Death Protein 1 (PD-1) inhibitors; 2) Programmed Cell Death Ligand 1 (PD-L1) inhibitors and ; 3) Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) inhibitors.[4] They are biologics known as monoclonal antibodies, which are synthetically produced proteins which mimic antibodies made in the body to fight cancer.[8] Recent studies have shown that this class of drug is only effective in a small percentage of patients with colon cancer, only about 15% (those with MSI-H/dMMR). It has been found that ICIs are mostly ineffective in patients with the more common form of metastatic colon cancer, MSI-L.[3] Therefore, it is imperative that more therapeutic options be made available to colon cancer patients so as to increase the likelihood of survival.

1.2.2 15-deoxy, $D^{12,14}$,-prostaglandin J^2-ethanolamide

The drug 15-deoxy, $D^{12,14}$,-prostaglandin J^2-ethanolamide (PMJ) is a hydrophobic anti-cancer drug derived from the endocannabinoid, arachidonoyl ethanolamide: this process is illustrated in Figure 2. PMJ exhibited the potential to treat both melanoma and colon cancer, as was demonstrated while conducting cytotoxicity and tumor treatment assays on mouse melanoma and colon cancer cells when it was being developed (see Figures 3 and 4). It is believed that the mechanism of action for PMJ is the induction of intense endoplasmic reticulum (ER) stress, promoting an unfolded protein response (UPR). UPR triggers apoptosis in cells, or programmed cell death. The drug also demonstrated potential for selective cytotoxicity towards cancerous cells, suggesting fewer adverse side effects than other anti-cancer drugs in use today.[9]

The delivery method for treating melanoma cancer with PMJ is, on the surface, fairly straight forward. With melanoma being a cancer of the skin, tumors are easily accessible and PMJ can be injected in the area of the tumor. This is not the case with colon cancer: here, PMJ would need to be administered intravenously in order to reach the colon. Here, the hydrophobicity of PMJ becomes an issue and necessitates the use of a delivery method that would increase the solubility of the drug. Thus, amphiphilic nanoparticles such as micelles can be of assistance.

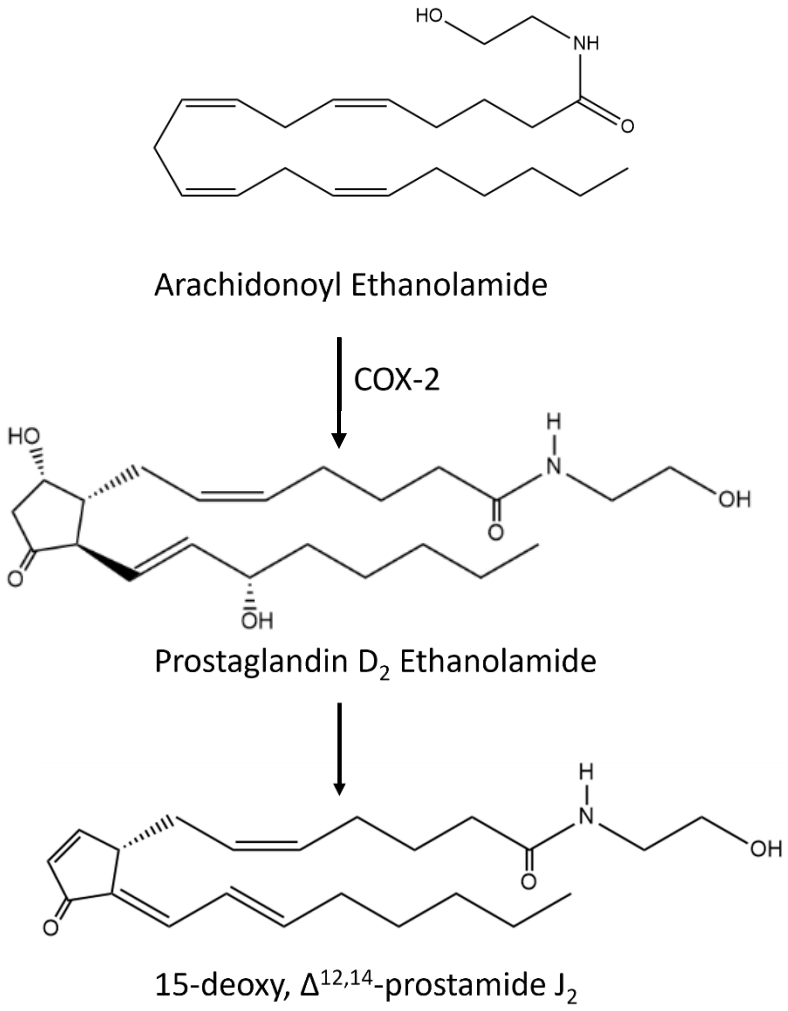

Figure 2 Metabolic derivation of PMJ from arachidonoyl ethanolamide.

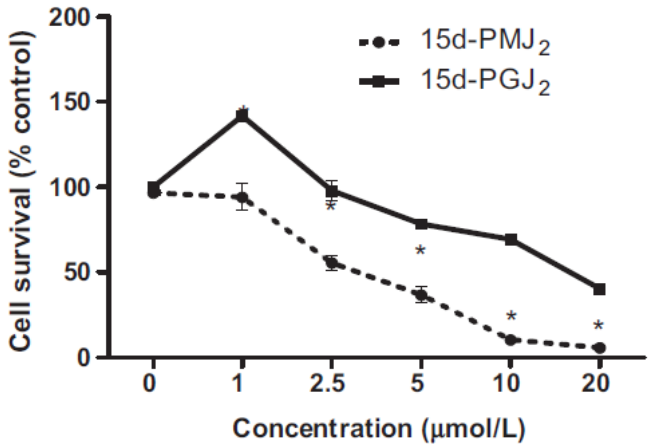

Figure 3 B16F10 cell viability after treatment with PMJ and PGJ for 24 hours at 37°C.[9]

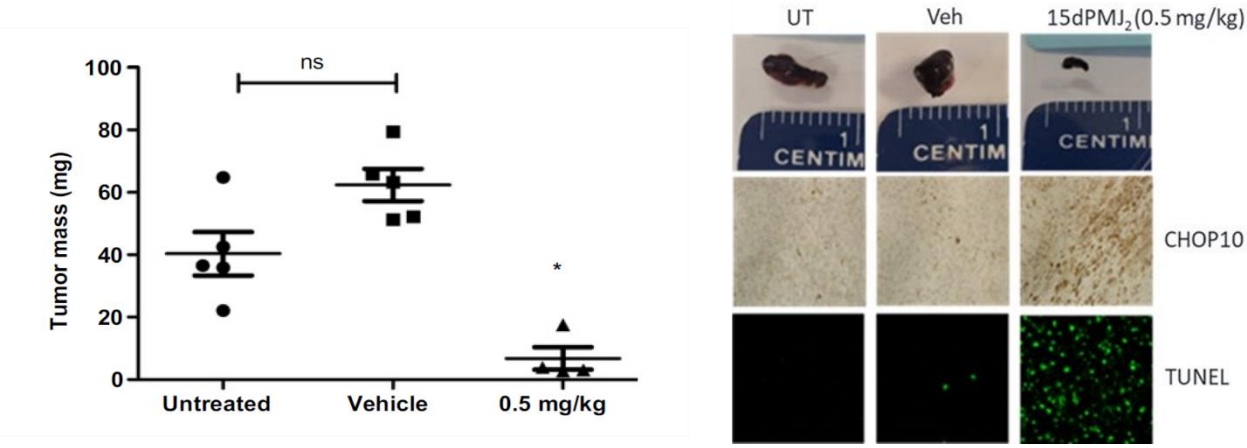

Figure 4 Data from B16F10 tumors that were untreated (UT) and treated with vehicle (Veh) and PMJ. In the graph on the right, ns means no significance.[9]

1.3 Micelles

Micelles are amphiphilic nanoparticles that have a core-shell structure that allows for the entrapment of different molecules. The classic structure includes a hydrophobic core and hydrophilic shell and is illustrated in Figure 5. This is an advantageous set-up as this allows hydrophobic drugs to become water soluble and thus safe for intravenous administration. Other desirable aspects of micelles are that they can be designed to be an appropriate size for tumor targeting (10-100 nm), have optimal stability and controlled drug release, as well as be

biocompatible for use in living organisms.[10,11] Therefore, micelles were deemed to be a viable solution to the issue of how to deliver a hydrophobic drug, such as PMJ, intravenously.

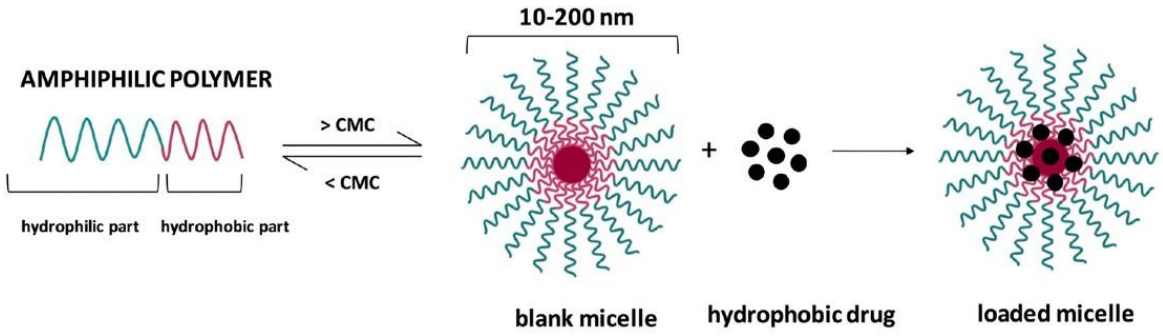

Figure 5 Formation and loading of a micelle.

1.3.1 DSPE and TPGS

Micelles made up of DSPE, whose figure is illustrated in Figure 6, (1, 2-distearoyl-sn-glycero-3-phosphoethanolamine polyethyleneglycol-2000) and TPGS, whose figure is illustrated in Figure 7, (D-α-tocopheryl polyethylene glycol 1000 succinate) have been studied previously by Jin et al. and others as nanocarriers for anti-cancer drugs.[12] Jin et al. found that the micelles had very low critical micelle concentrations (CMC) as low as 0.071 g/L, high encapsulation efficiency (EE) above 85%, and an appropriate size for tumor treatment around 18.5 nm (the ideal size for nanoparticles used in tumor treatments is between 10-100 nm). These qualities are important in micelles because they demonstrate that they are stable at low concentrations, can hold the majority of drug introduced to them, and they are small enough to reach tumors and be retained there. They also found that the micelles themselves were minimally toxic to cancer cells, and when loaded with anti-cancer drug Asulacrine (ASL) they were comparatively toxic to cancer cells as free ASL in solution. The micelles also demonstrated long-term stability over a

30-day period.[12] However, other micelle formulations were also investigated for use as a delivery system during the project.

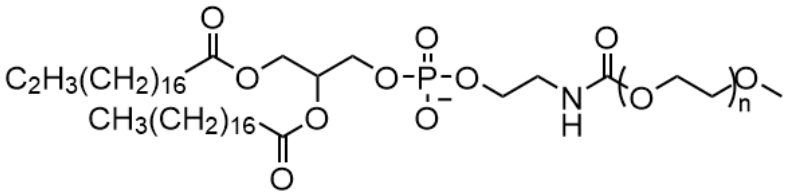

Figure 6 Structure of DSPE.

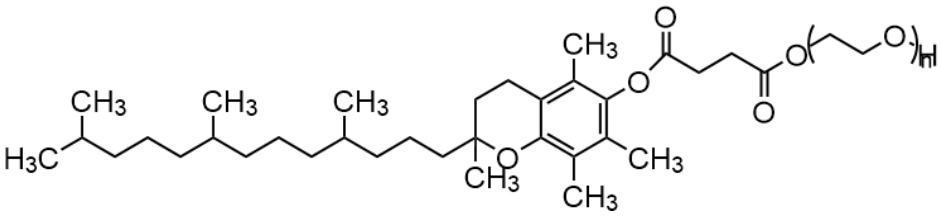

Figure 7 Structure of TPGS.

1.3.2 Pluronic F127

Pluronic F127 micelles are made up of water-soluble tri-block copolymers consisting of polyethylene oxide (PEO) and polypropylene oxide (PPO) where F127 has block lengths of $(PEO)_{100}$-$(PPO)_{65}$-$(PEO)_{100}$ (see Figure 8 for structure) . It can self-organize to form micelles with PPO making up the core and PEO making up the surface.[13] One of the main reasons it is being considered is due to its history of use in loading and delivering fluorescent dyes and proteins. It demonstrated a low CMC of around 0.0031%, a particle size of 20.73 ± 0.16 nm was measured using DLS, and low cytotoxicity to brain capillary endothelial cells, as well as an ability to work well when paired with other amphiphilic polymers such as TPGS.[14]

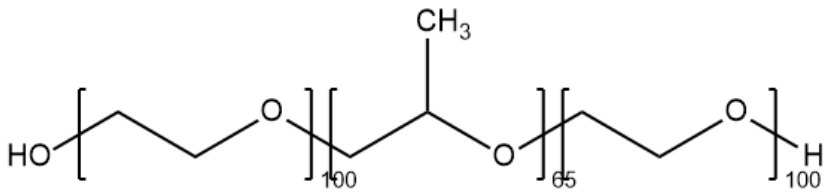

Figure 8 Structure of pluronic F127.

1.4 Project Goals

There are two main goals for this research project. The first is the physical characterization of a suitable micelle formulation: the CMC must be determined to ensure all formulations are made and maintained above this value, the EE must be measured to establish the amount of encapsulated drug that will be delivered, and the size needs to be between 10-100 nm to best enable the micelles to reach their tumor targets. The second is a viability assay to determine the cytotoxicity of empty and drug loaded micelles on mouse colon cancer cells (CT26) cells and mouse melanoma cancer cells (B16F10). The currently approved standard of care drug for colon cancer, irinotecan, will be used as a positive control for comparison with the efficacy of 15-deoxy, $\Delta^{12,14}$-PMJ_2.

Chapter 2: Experimental Methods

2.1 Preparation of DSPE:TPGS Micelles

The micelle former stock was made by dissolving DPSE and TPGS, using a 1:5 molar ratio of DSPE:TPGS, in ethanol. The micelles were then made into a thin film by evaporating the ethanol off using a rotavapor at 45°C. The film was then rehydrated using either DI water, 1x PBS pH 7.4, or serum free RPMI 1640, depending on the experiment being conducted. This solution was warmed in a water bath at 45°C for five minutes and vortexed for 30 seconds (the water bath and vortexing were repeated twice).

2.1.1 Preparation of Loaded DSPE:TPGS Micelles

Once the micelles were dissolved in ethanol and the desired quantity was delivered to a scintillation vial, the micelles were loaded with the desired molecule (fluorophore, drug, etc). The solution was then rotovapped at 40°C, leaving a thin film. The thin film was then rehydrated in with DI water, 1x PBS pH7.4, or serum free RPMI 1640, at which point the solution was warmed in a water bath at 45°C for 5 minutes and vortexed for 30 seconds, twice. The rehydration and subsequent warming in the water bath followed by vortexing prompted the molecules to be loaded into the micelles to become entrapped.

2.2 Preparation of PMJ

The PMJ used throughout the project was synthesized in lab and stored at -20°C. To synthesize PMJ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ) (Cayman Chemical, Ann Arbor, MI) was

rotovapped to remove methyl acetate, leaving a yellow oil. In a solution of DMF, the PGJ was then combined with 1.5 eq O-(benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), after which 3.0 eq of diisopropylethylamine (DIEA) was added. After five minutes of stirring, 3.0 eq of ethanolamine was added and the solution was left to stir for two hours under nitrogen. The product was then separated from the byproducts of the reaction via liquid-liquid separation using water and ethyl ether. The product was then purified using a chromatography column and product identity was confirmed using TLC. The structure of the product was determined using H^1 NMR, and the stereo configuration was confirmed using VCD (see Appendix: Structural Determination of 15-deoxy, $D^{12,14}$-prostaglandin J_2-ethanolamide).

2.3 Preparation of Pluronic F127

Pluronic F127 micelles were dissolved in acetone, after which the micelle solution was then added to an aqueous solution (DI water, PBS, or serum free DMEM/High Glucose) at a volume ratio of 1:1 aqueous solution to acetone. The acetone was then removed using a rotavaporator, at which point micellization occurred.

2.3.1 Preparation of Loaded Pluronic F127

After the micelles were dissolved in acetone, then the micelles could be loaded with the desired molecule (fluorophore, drug, etc). The micelle and desired molecule were then added dropwise to an aqueous solution, at which point the micelles self-assembled and entrapped the molecule of interest. The acetone was then removed via rotavaping.

2.4 CMC

Critical micelle concentration (CMC) is the concentration above which micelles will form. This is an important characteristic of micelles as this value denotes the stability of the nanoparticle and determines the concentrations at which the micelles can be made. The lower the CMC, the more stable and resistant to dilution the micelles are, which is an important consideration when thinking about intravenous delivery of the drug loaded micelles for colon cancer treatment.

2.4.1 Fluorescence Instrumentation

Fluorescence was the chosen method for quantifying CMC for the micelle formulations. A Photon Technology International (PTI) fluorometer was used for this project. The fluorometer works by shining a laser at a specific excitation wavelength (dependent on the fluorescent probe used) through the sample, and recording the emission given off by the sample as counts per second using a detector. The detector then sent the recorded data to a computer which interpreted it using PTIFelixX32 software.

2.4.2 Method for CMC Determination

A series of micelles loaded with the fluorescent probe pyrene were made with half the concentrations being above the predicted CMC and the other half being below the predicted CMC. Fluorescence was performed on these concentrations using an excitation wavelength of 340 nm and an emission range between 355-500 nm. The data was then analyzed using Excel by plotting the log of the concentrations used vs. counts per second at the maximum absorbance at 393 nm. The inflection point in the plot was determined by fitting a linear function separately to

the high and low concentration values and taking the anti-log of the crossing point as an estimate of the CMC value.

2.5 Determination of Encapsulation Efficiency

The encapsulation efficiency (EE) of a micelle is the ratio of drug initially introduced to the sample to the amount of drug entrapped by the micelle. This was calculated by performing HPLC on drug loaded micelles, one set of which was dialyzed (dialyzed sample, DS), while the other was not dialyzed (standard sample, SS). The EE was represented as a percentage (see Equation 1), with the area under the curve for three DS samples being divided by the of three sets of SS samples. These values were then converted to percentages and averaged.

$$\frac{DS}{SS} \times 100\% = \%EE \qquad \text{Equation 1}$$

2.5.1 HPLC Instrumentation

High performance liquid chromatography (HPLC) was used to determine the amount of the drug present in micelle samples. A Bio-Rad HPLC instrument was used alongside the Chrom-Lab analysis program. Reverse phase HPLC was performed on the samples (n=3) using a 5 µm C18 column with the dimensions 250 × 4.6 mm, and DI water and HPLC grade acetonitrile both treated with 0.1% TFA (trifluoroacetic acid) as the solvents. The flow rate was 1 mL/min, and four wave lengths (210, 221, 255, and 360 nm) were monitored using a multi wave UV-Vis detector. Figure 9 depicts a typical HPLC set up, showing that the sample was injected into the injection port, pumped through the column using a solvent gradient so as to separate the micelles from the entrapped drug, and passed through the multi-wave UV-Vis detector and exposed to various wavelengths of light, one of which is where the drug of interest emits. For irinotecan,

this was at 360 nm, and for PMJ it was 229 nm. The data was then recorded and analyzed using ChromLab.

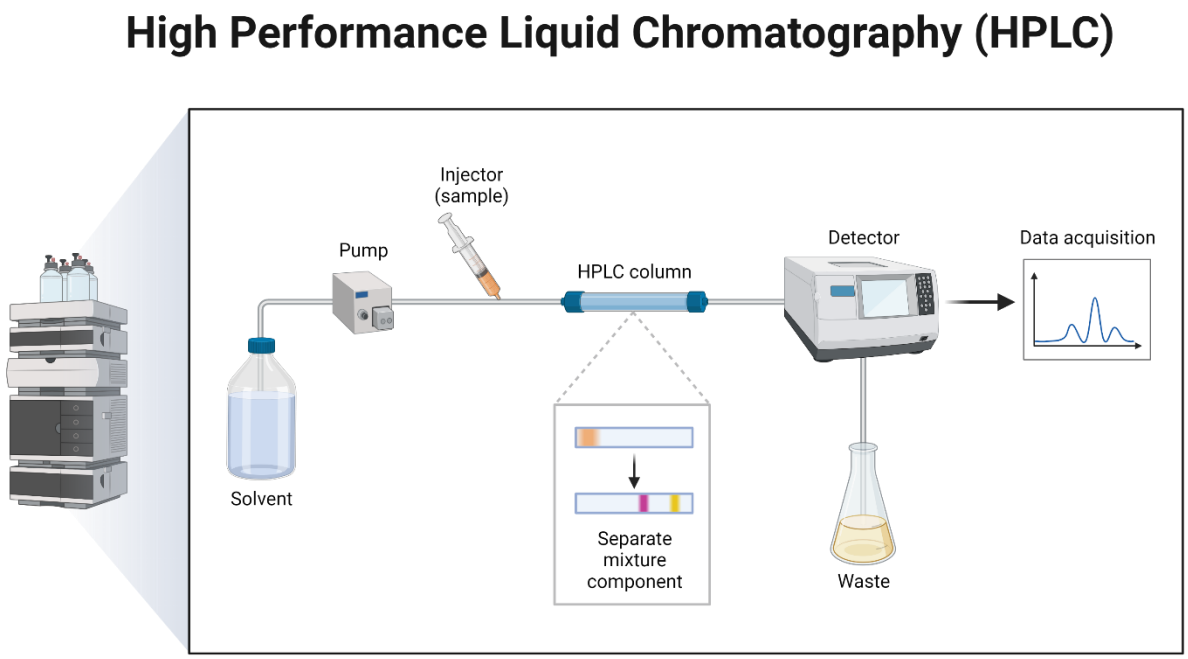

Figure 9 HPLC instrument diagram

2.5.2 Method for EE Determination

EE was determined by loading micelles with the drug of interest as per preparation of loaded micelle sections. Multiple aliquots of pairs of the rehydrated solution were made, half of which were dialyzed in DI water for two hours to ensure any free drug remaining in solution was removed and the other half were used as standards. Then, for loaded DSPE:TPGS micelles, the samples were freeze-dried overnight and made into solution once more using HPLC grade acetonitrile. For loaded Pluronic F127 micelles, freeze-drying was not necessary. The data was then analyzed, using a calibration curve made from various concentrations of the drug of interest, in Excel.

2.6 Micelle Size

The size of micelles is important due to the need for them to be able to penetrate tumors to deliver entrapped drug. The proposed appropriate size range suggested by Jin et al. was 10-100 nm and thus is a part of the criteria that suitable micelles must meet for this project.[12] Another aspect of the size of micelles is the homogeneity of the micelle particles in solution, this can be measured as the polydispersity index (PDI). That is, it needed to be determined whether there were multiple populations of varying sizes within a sample. Both TEM and DLS were used to ascertain these characteristics.

2.6.1 Instrumentation for TEM

Transmission electron microscopy (TEM) directs beams of electrons towards samples that are either embedded in a resin, stained, or cryofixed, and captures images of the structures present in the samples.[15] This is achieved due to areas of electron density in the sample absorbing more of the electron beam, while less dense areas allow the beam to pass through more easily. The intensity of the electron beam is recorded using a detector, which transforms these intensities into a comprehensive image of the nanoparticles present in the sample.

2.6.2 Method for TEM

Micelle samples were prepared according to their experimental methods in lab and sent to UNC Chappel Hill for imaging. There, the team at UNC dehydrated the micelle samples, stained them with 2% uranyl acetate, and recorded images of the micelles in solution. These images denoted the size of the micelles and assisted in further characterizing the formulations made throughout the project.

2.6.3 Instrumentation for DLS

Dynamic light scattering (DLS) is a technique that can determine the size of molecules, as well as determine whether a sample has multiple populations of differing molecular sizes. This works by directing a laser from a light source at a sample and measuring the intensity of the light scattered by the refraction of the particles over time in the sample using a detector. The size and dispersity of the particles in solution are calculated using the Stokes-Einstein equation (Equation 2) and by creating and using the autocorrelation function (ACF). In the Stokes-Einstein equation, D is the diffusion coefficient, k_B is the Boltzmann constant, T is temperature, **η** is the dynamic viscosity and r_H is the hydrodynamic radius of the particle.[16]

$$D = k_B T / 6\pi\eta r_H \qquad \text{Equation 2}$$

The ACF is the mathematical relationship between the diffusion coefficient of the particles and the variation of the intensity of their scattered light over time. Essentially, the intensity of the particles is recorded over time which creates a decay function, where the size of the particle influences the rate of decay. The particles behave according to Brownian motion, and smaller particles move faster than larger particles; this leads to the rate of decay being quicker for smaller particles than for larger particles. The data obtained from DLS can then be fit to the ACF in order to determine the intensity distribution of the particles in solution, thus allowing for the elucidation of both the size of the particles, as well as the size distribution, aka the number of different size populations within a solution. This is referred to as the polydispersity index (PDI), which is calculated by taking the standard deviation of the hydrodynamic radius of the particles in solution. Should a solution of particles have a PDI of less than 0.3, then it is considered mostly monodisperse. A PDI of between 0.3 and 0.7 is somewhat polydisperse, and above 0.7 it is highly polydisperse.[16]

2.6.4 Method for DLS

Micelle samples (both loaded and empty) were prepared and analyzed using the Malvern DLS instrument. The cuvette material used for micelle samples was polystyrene latex, and the measurement angle used for all experiments was 90°. Each sample was measured three times, and each measurement consisted of 25 scans, with a run duration of 10 seconds for each scan.

2.7 Cytotoxicity

Cytotoxicity assays are used to determine how toxic certain molecules or drugs are to cells. In this project, the cell lines used were CT26 and B16F10. CT26 cells are mouse colon cancer cells while B16F10s are mouse melanoma cancer cells. The cytotoxicity assay used in this project was the MTS assay. This assay is a color changing assay that indicates the percentage of living cells present in an experiment. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) reagent is reduced to formazan in the presence of NAD(P)H-dependent oxidoreductase enzymes expressed in the mitochondria of living cells.[17] This results in a change in color of the solution from yellow to dark brown/red. The absorbance of this color change can then be recorded and the data analyzed to determine the percentage of living cells present after treatment with a compound or drug of interest.

2.7.1 Plate Reader Instrumentation

A plate reader uses UV-Vis to record the absorbance of the color change from the reaction between MTS reagent and NAD(P)H-dependent oxidoreductase enzymes. The instrument used in this project was an Infinite M200 Pro and the software used to record the absorbance from the plate was Tecan i-control.

2.7.2 Method for Cytotoxicity Determination

The cytotoxicity of both drugs and micelles was determined by performing an MTS assay on both the B16F10 and CT26 cancer cell lines. The cells were plated on a 96-well plate and left to incubate for 48 hours at 37°C to achieve a confluency of around 70%. They were then treated with the solution of interest and left to incubate at 37°C for 24 hours. The cells were then treated with MTS and left to incubate for one hour at 37°C, after which the absorbance was obtained at 490 nm using a plate reader and the data was analyzed via Excel.

Chapter 3: Experimental Results for 1:5 DSPE:TPGS Micelle Formulation

3.1 CMC

The CMC for 1:5 DSPE:TPGS empty micelles was determined to be 0.006 ± 0.002 (n=3 for mean ± SD) mg/mL (4.06 μM).[18] The concentrations used to determine CMC were based on previous literature findings from Jin et al.[12] and thus ranged from 0.0001 mg/mL (5.8 E-05 mM) to 0.30 mg/mL (0.17 mM). The data from one fluorescence-based CMC experiment is shown in Figure 10.

Multiple research groups have worked with DSPE:TPGS, one of which was Zhang et al., who reported the CMC of 1:5 DSPE:TPGS as 7.2 μg/mL via fluorescence using pyrene as a probe.[10] This value is compared very closely to the one determined during the research project. The CMC obtained by Jin et al. for empty micelles was 0.117 mg/mL (0.048 mM), which is two orders of magnitude higher than was obtained from the samples prepared for this study.[12] This could be due to the difference in methods for obtaining CMC between the two studies with Jin et al. using the drop weight method while this project used fluorescence. The drop weight method is based on the balance between the drop weight of the micelle solution and the surface tension. As the concentration gets closer to that of the CMC, the less the surface area changes.

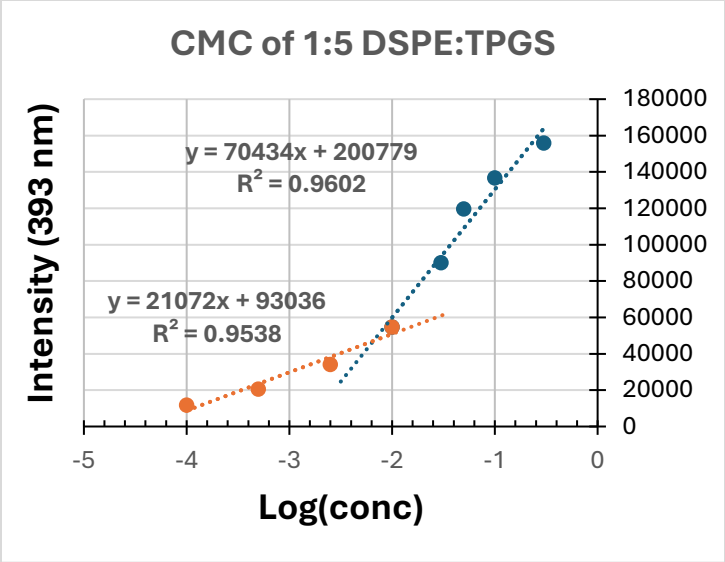

Figure 10 CMC analysis of fluorescent data obtained from pyrene loaded micelles using a range of concentrations from 0.0001 mg/mL to 0.30 mg/mL.[18]

3.2 Encapsulation Efficiency

Irinotecan loaded 1:5 DSPE:TPGS were prepared at a 1:6 molar ratio of drug:micelles, and EE experiments were carried out in triplicate to ensure confidence in the results obtained. The average EE for irinotecan loaded micelles was around 75.4 ± 2.1 (n=3 for mean ± SD) %. PMJ loaded micelles were also prepared at a 1:6 molar ratio of PMJ:micelles with EE experiments carried out in triplicate. The average EE for PMJ loaded micelles was around 85.6 ± 8.1 (n=3 for mean ± SD)%.[18] These EEs are similar to those from other studies that used DSPE:TPGS micelles for drug delivery. Zhang et al. and Jin et al. used DSPE:TPGS to deliver alantolacetone and quercetin, and Asulacrine (ASL) respectively, and their EEs ranged from 86 to 94%.[10,12]

3.3 Micelle Size

An empty micelle solution with a concentration of 0.30 mg/mL (0.17 mM) was made in DI water and sent to UNC Chapel Hill to perform TEM on the solution to ascertain the average size of the micelles. Image A in Figure 11 depicts the micelles at 100,000x magnification and image B depicts them at 500,000x. By visual inspection, the average size of the micelles was found to be around 25 nm. However, as can be seen in image B (see Figure 11B), there are several smaller micelles. Thus, it can be assumed that there are several size populations of micelles in solution.

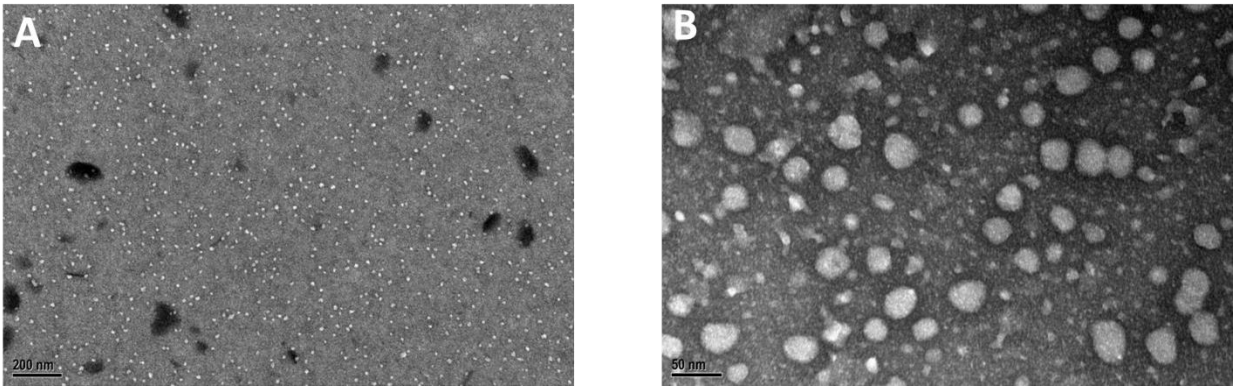

Figure 11 Image A depicts empty micelles captured by TEM at 100,000x. Image B depicts empty micelles magnified to 500,000x.

DLS was used as another method to determine micelle size and PDI (Table 1). Samples of irinotecan loaded micelles and empty micelles (irinotecan at 500 μM and micelles at 5 mg/mL or 2.9 mM) prepared in PBS were sent to the Beltran-Huarac lab at the Brody School of Medicine (samples run by Yara Maavah). The mean sizes of the loaded and unloaded micelles were determined to be 14.42 nm and 14.71 nm respectively, with polydispersity indexes (PDIs) of 0.21 and 0.19. Loading irinotecan into the micelles seems to reduce the diameter of the

micelles slightly, but there is little change otherwise between loaded and empty micelles at this concentration of irinotecan. Two 5 mg/mL samples, one in DI water and the other in PBS, were analyzed using DLS. The average size of the micelles prepared in water was around 5 nm while the micelles prepared in PBS were around 15 nm. The PDIs of these measurements were 0.29 and 0.048, respectively. The sizes of micelles prepared in PBS were consistent across experiments, all averaging in size at about 15 nm, and all had PDIs that were less than 0.3, indicating mostly monodisperse solutions of micelles. For comparison to the sample prepared in DI water, a 1.0 mg/mL solution of 1:5 DSPE:TPGS micelles in DI water analyzed using an instrument provided as a demo from Malvern (Zetasizer Ultra Red). These micelles were found to have a diameter of about 5 nm and a PDI of 0.220.

Figures 12 through 15 depict the DLS data noted in Table 1, and as can be seen by the various peaks present in the spectra alongside the PDIs for the samples, there are some smaller populations of various particle sizes present in the majority of the samples.

Table 1. DLS Data from Beltran-Huarac Lab & Brody School of Medicine

System	Solvent	Concentration (mg/mL)	Mean Size (nm)	Polydispersity Index
Empty Micelles	Water	5	4.87 ± 0.04	0.29 ± 0.03
Empty Micelles	Water	1	5.52 ± 0.13	0.220 ± 0.038
Empty Micelles	PBS	5	15.06 ± 0.2	0.048 ± 0.011
Empty Micelles	PBS	5	14.71	0.198
Irinotecan + Micelles	PBS	5	14.24	0.212

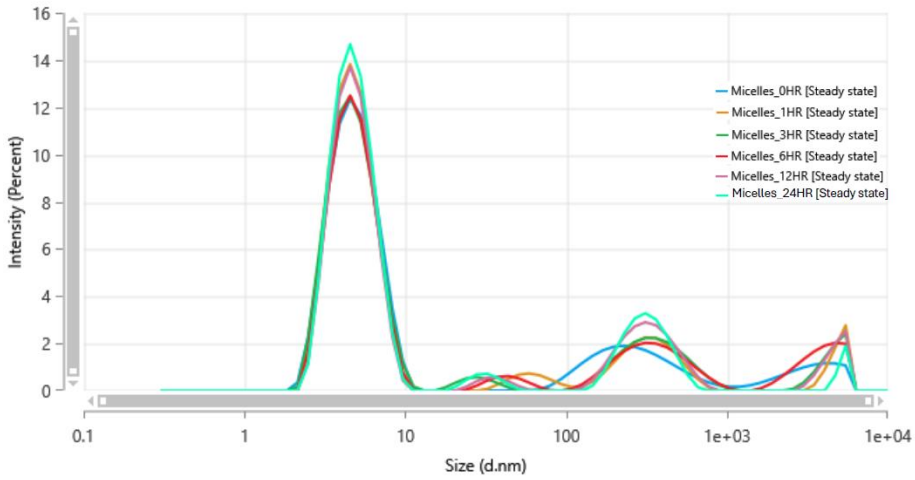

Figure 12 Time dependent DLS data made by Yara Maayah for 1:5 DSPE:TPGS at 5 mg/mL in DI water. Size populations were around 5 nm and 340 nm with a PDI of 0.29. Data representative of one independent run.

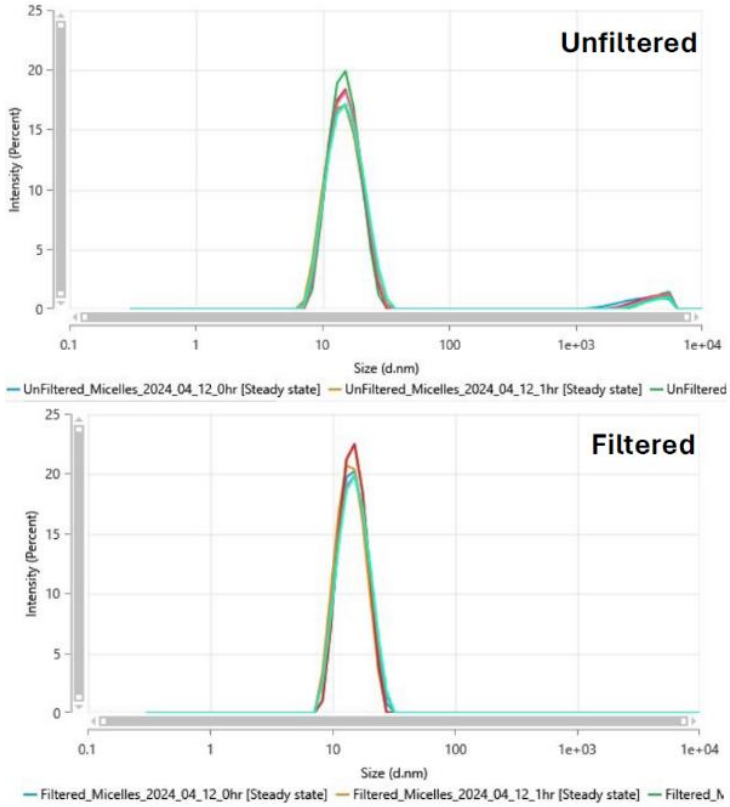

Figure 13 Time dependent DLS data made by Yara Maayah of 1:5 DSPE:TPGS at 5 mg/mL in 1x PBS pH 7.4 with size populations around 15 nm and 4169 nm in the unfiltered sample and 15 nm in the filtered sample. Data representative of one independent run, each.

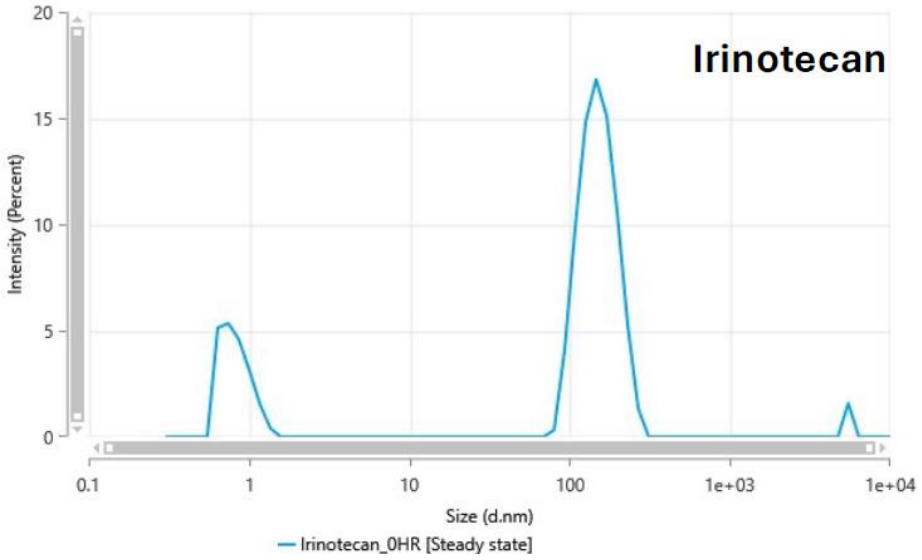

Figure 14 DLS data made by Yara Maayah of irinotecan HCl at 0.312 mg/mL in 1x PBS pH 7.4 with size populations around 1 nm and 154 nm. Data representative of one independent run.

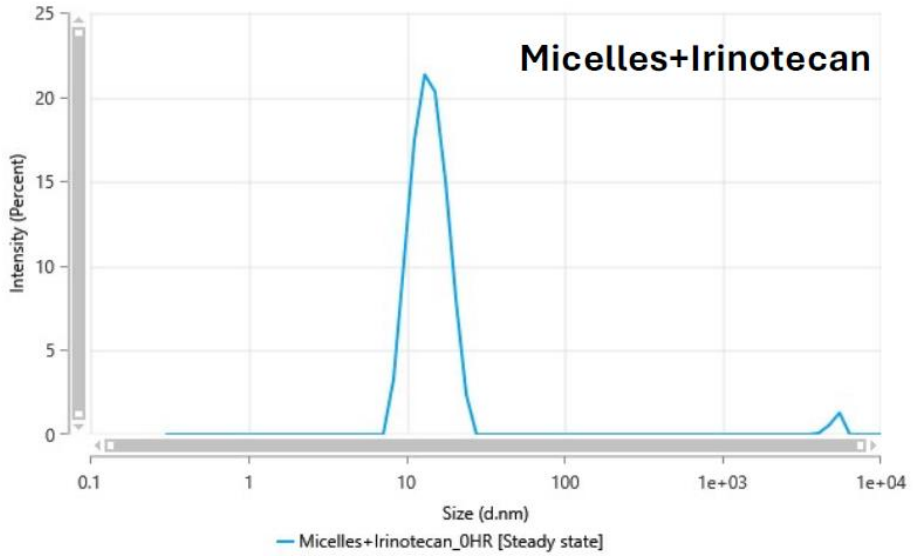

Figure 15 DLS data made by Yara Maayah of irinotecan loaded 1:5 DSPE:TPGS micelles at 0.312 mg/mL irinotecan and 5 mg/mL 1:5 DSPE:TPGS in 1x PBS pH 7.4 with size populations around 14 nm and 5179 nm. Data representative of one independent run.

3.4 Cytotoxicity

The end goal of the cytotoxicity assays was to determine the cytotoxicity of drug loaded micelles. Therefore, an MTS assay of free irinotecan HCl (0.006 mg/mL-0.312 mg/mL, or 10-500 µM) treated CT26 cells was carried out for comparison to irinotecan HCl loaded micelles, as seen in Figure 16. At the highest concentration of 500 µM irinotecan HCl, the cell viability was less than about 10%. This value is in line with that found by Evans et al. where they observed the cell viability of CT26 cells to be around 10% with 300 µM irinotecan.[19]

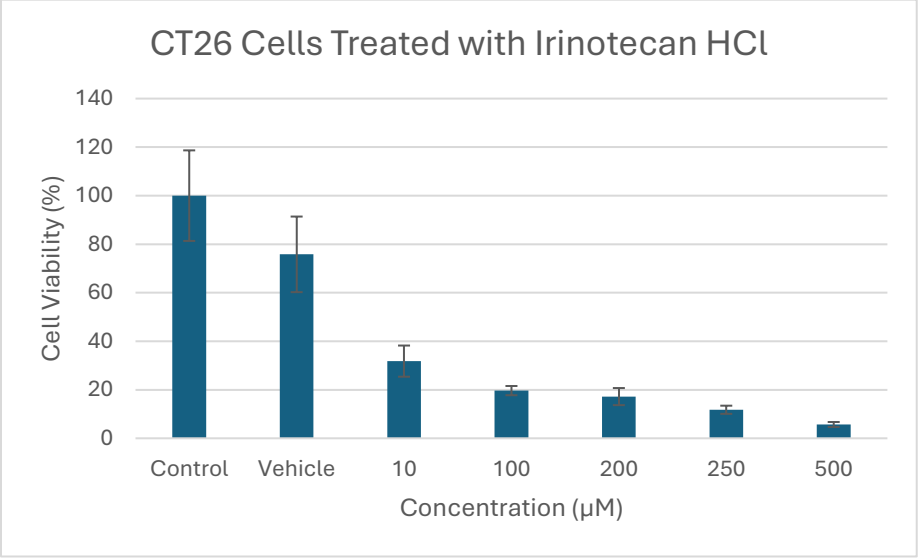

Figure 16 Cell viability of CT26 cell line treated with free irinotecan and assessed using an MTS assay. Data representative of the mean ± SD of three independent experiments.

The cytotoxicity of empty micelles was then obtained using concentrations ranging from 0.1 mg/mL to 5 mg/mL (0.058-2.9 mM). Multiple assays were completed, all of which resulted in significantly decreased cell viability. It was thought that the CT26 cells were too easily detached from the plates in the presence of micelles. So, a more robust cell line known as B16F10 was then treated with the same empty micelle preparations. However, the results were the same as before.

Finally, after conducting a 24-hour time and concentration dependent assay using empty micelles ranging in concentration from 0.01 mg/mL to 0.2 mg/mL (0.0058-0.12 mM), it was determined that the micelle formulation was cytotoxic. The results of this assay are depicted in Figure 17.

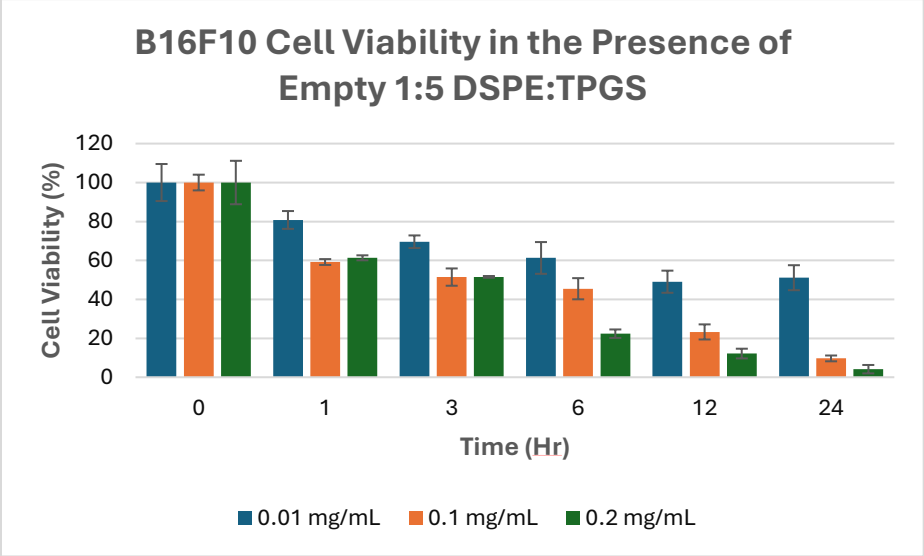

Figure 17 B16F10 cells treated with empty 1:5 DSPE:TPGS micelles ranging in concentration from 0.01 mg/mL to 0.2 mg/mL. Data representative of mean ± SEM of four wells per concentration in one independent experiment.

Further testing using dose curves for DSPE and TPGS, respectively, revealed TPGS to be the cytotoxic aspect of the micelles. Treatment of the B16F10 cells with as low as 0.05 mg/mL of TPGS resulted in a cell viability below 20%. These results can be found in Figure 18. At this point, it was decided that alternative micelle formulations would be investigated to find a suitable formulation to deliver PMJ.

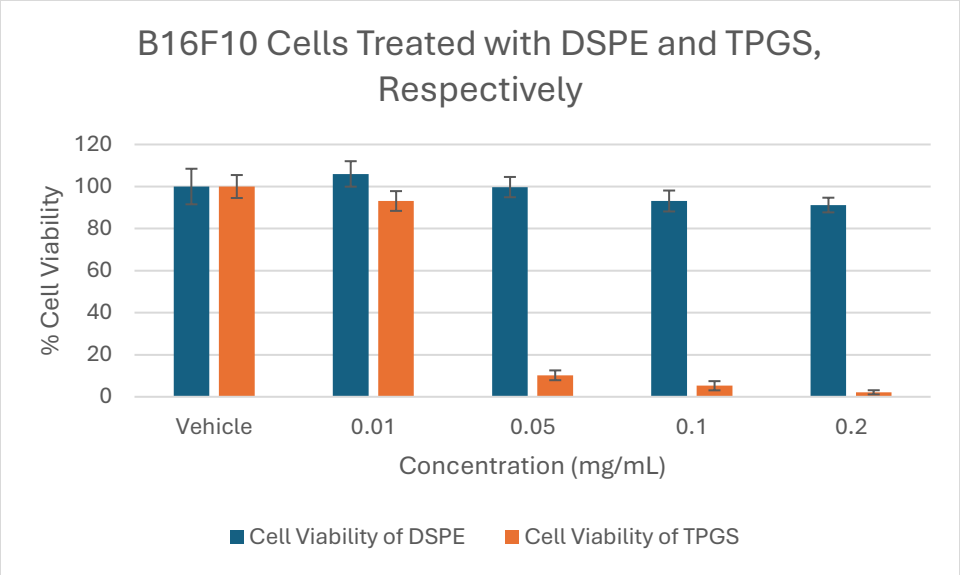

Figure 18 B16F10 cells treated for 24 hours with DSPE and TPGS, respectively, at various concentrations and analyzed via MTS and Excel. Data representative of mean ± SEM from three independent experiments

Chapter 4: Experimental Results for Pluronic F127

4.1 CMC

The CMC for Pluronic F127 empty micelles was determined to be 0.743 ± 0.151 (n=3 for mean ± SD) mg/mL (0.063 mM). The initial concentrations used to determine CMC were based on previous literature findings from Alexandridis et al,[13] who found the CMC to be around 7 mg/mL (0.555 mM) at 25 °C.[13] The CMC obtained here compared to that of Alexandridis is much smaller at around the same temperature, and thus the concentrations used to obtain CMC in this project were adjusted. However, the group in Alexandridis et al. dissolved their fluorescent probe DPH in methanol; they stated that there was about 1% methanol present in samples of Pluronic F127 made for CMC, and that other research groups had reported that 1% methanol could increase CMC by 40%.[20] The concentrations investigated here ranged from 0.1 mg/mL to 20 mg/mL (0.008-1.59 mM), and the data from one fluorescence-based experiment is shown in Figure 19.

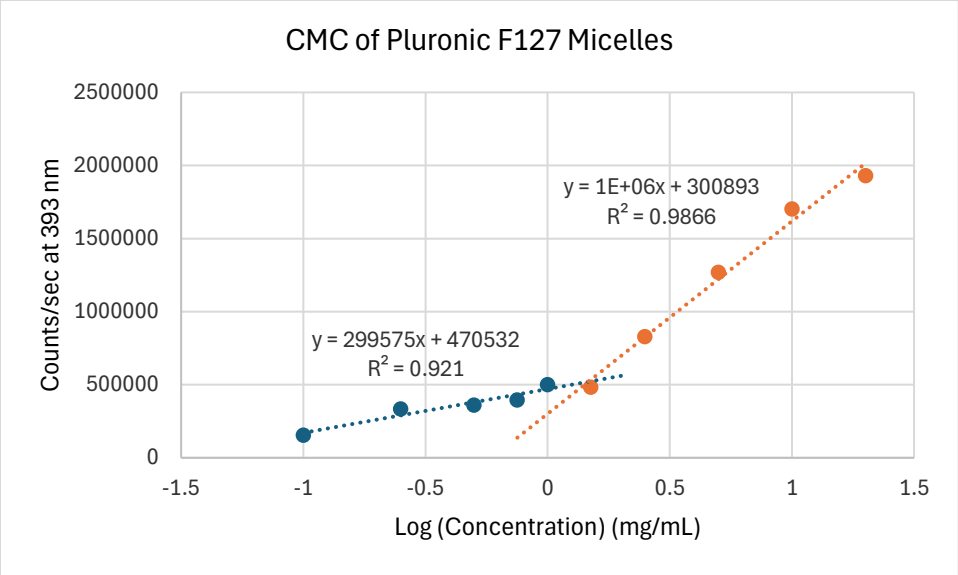

Figure 19 Fluorescent data for different concentrations of Pluronic F127 loaded with 0.5 μM pyrene at room temperature.

Other studies that have used Pluronic F127 have found similar CMCs, including Butt et al. who found the CMC of F217 to be around 0.63 mg/mL (0.5 mM) at 37°C.[21] However, instead of using either fluorescence or the drop weight method, they used DLS to determine micelle formation. Molar concentration was graphed against intensity, and the concentration at which there was a sudden increase was determined to by the CMC. Overall, the CMC measured here agrees well with values reported in the literature.

4.2 Encapsulation Efficiency

The encapsulation efficiency of Pluronic F127 micelles loaded with irinotecan HCl was determined to be 81.5 ± 4.3 (mean ± SD, n=3)% . The average concentration of irinotecan HCl that remained entrapped in the micelles was calculated to be 377 μM, or 0.235 mg/mL. This was determined by loading micelles at a concentration of 10 mg/mL (0.79 mM) with irinotecan HCl at a concentration of 0.312 mg/mL (500 μM). A calibration curve was created using HPLC data obtained from free irinotecan in DI water at concentrations ranging from 100-500 μM (0.062-

0.312 mg/mL, see Figure 20). Example chromatograms of drug loaded micelles are depicted in Figures 21 and 22, showing a standard drug loaded micelle and a dialyzed sample, respectively. Retention times for irinotecan were found to vary by a minute or two between trials, possibly due to differences in room temperature and atmosphere day to day. This variation could have possibly been reduced by using a column heater at a constant temperature of 25°C, but our instrument lacks this feature. Nonetheless, instead of using a specific retention time to analyze the data, the peak representing irinotecan was identified and followed between trials.

Since encapsulation efficiencies for a single micelle preparation vary depending on the identity of the drug and the amount introduced to the micelle, this makes it difficult to make a direct comparison of these values. For example, in F127 loaded with docetaxel (MW = 807.9 g/mol) has EEs ranging from 46 to 65% for drug/copolymer ratios between 0.3 to 0.05 weight percent (w/w%); F127 loaded with doxorubicin (MW = 543.5 g/mol) has EEs ranging from 41 to 60% for drug/copolymer ratios between 0.3 to 0.05 weight percent (w/w%).[22] For comparison, the 81.5% EE for irinotecan loaded micelles was measured using a drug/copolymer ratio of 0.024% w/w%, which is lower than the ratio used in the study cited above (i.e. 0.05). Nevertheless, this value fits the trend of observing a higher EE for lower drug/copolymer ratios.

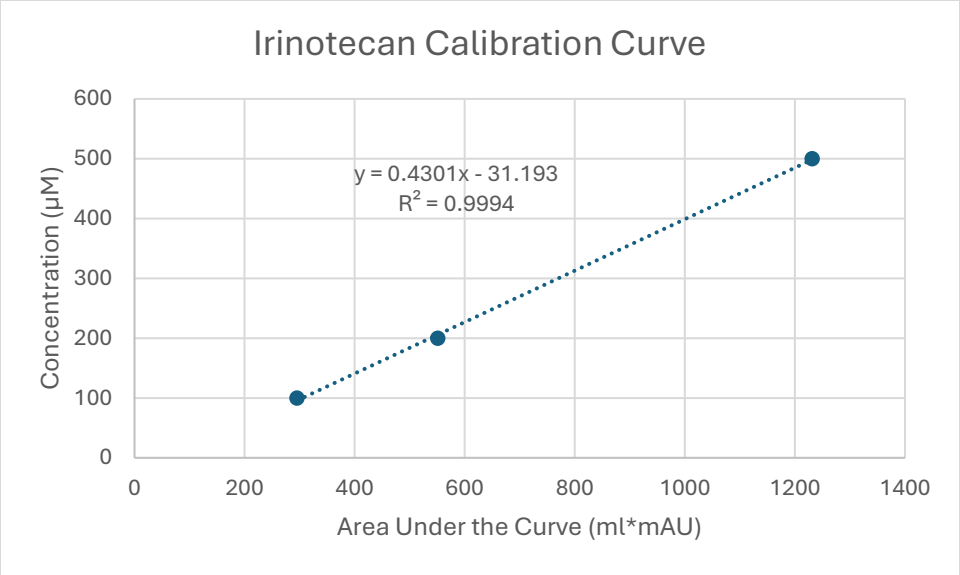

Figure 20 Calibration curve for irinotecan HCl loaded Pluronic F127 micelles measured at 360 nm, with irinotecan HCl concentrations ranging from 100 µM to 500 µM.

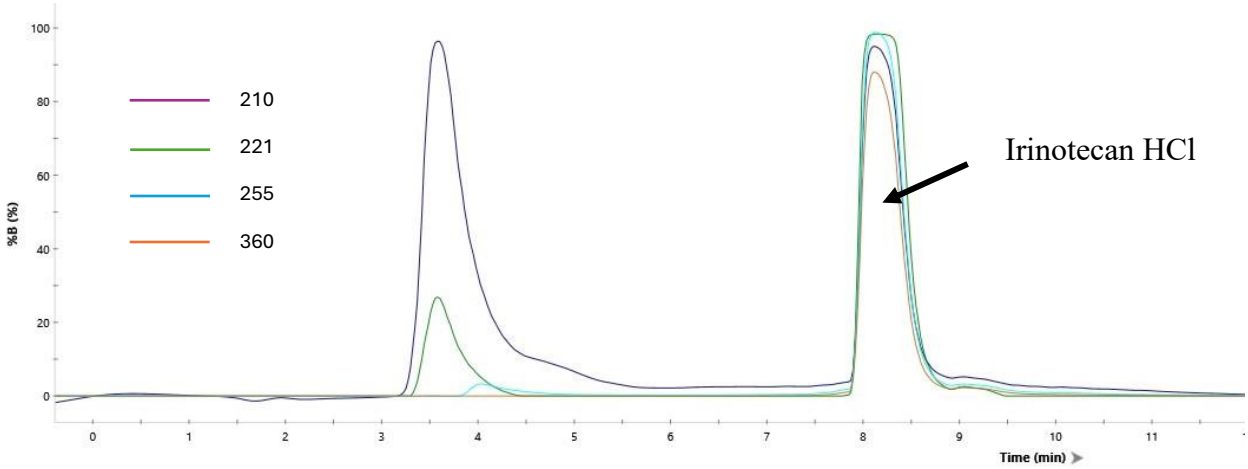

Figure 21 Chromatogram depicting a standard sample of irinotecan HCl (0.312 mg/mL) loaded Pluronic F127 micelles (10 mg/mL).

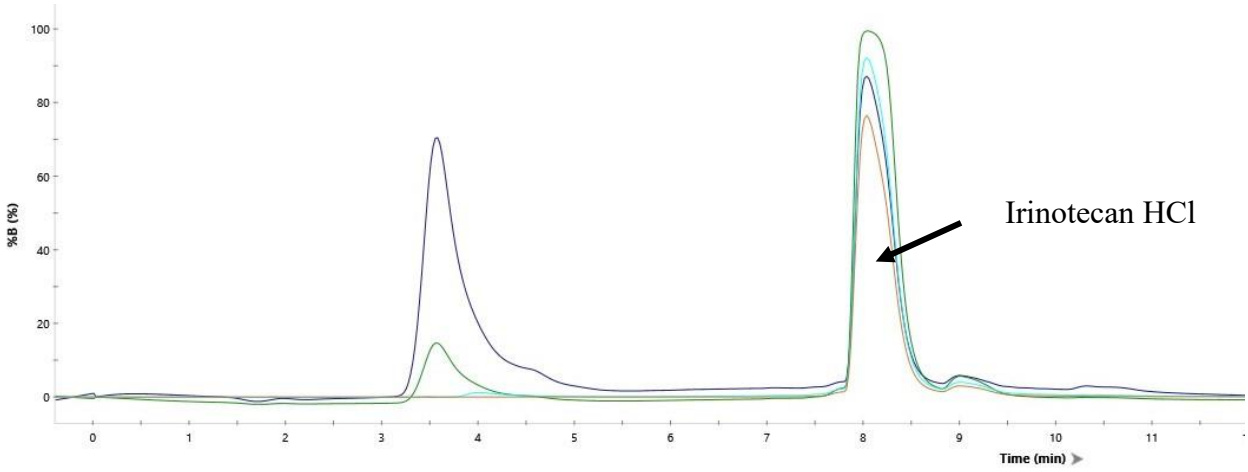

Figure 22 Chromatogram depicting a dialyzed sample of irinotecan HCl (0.312 mg/mL) loaded Pluronic F127 micelles (10 mg/mL).

4.3 Micelle Size

The size of both empty and drug loaded micelles was determined, as well the size of empty micelles made in DI water (pH 5.5) and in PBS (pH 7.4). The concentration of Pluronic F127 micelles used was 25 mg/mL (1.98 mM). The concentration of irinotecan used was 0.312 mg/mL (500 μM). The average size of empty micelles made in DI water was 13.2 nm with an average PDI of 0.428 (n=4), while the empty micelles made in PBS were 23.2 nm with an average PDI of 0.337 (n=4). The irinotecan loaded micelles made in PBS had an average size of 18.6 nm and an average PDI of 0.557 (n=4). Table 2 depicts the data obtained, where the size of the micelles was expressed as the mean ± SD. All the PDIs from each micelle variation had a PDI between 0.3 and 0.6, indicating that the solutions were at least moderately polydisperse. Figures 23 through 25 depict the spectra from the F127 DSL data, and as can be seen by the various peaks present in the spectra alongside the PDIs for the samples, there is usually one other size population present in the data.

Table 2. DLS Data for Pluronic F127 Micelles.

System	Solvent	Concentration (mg/mL)	Mean Size (nm)	Polydispersity Index
Empty Micelles	Water	25	13.17 ± 2.46	0.428 ± 0.070
Empty Micelles	PBS	25	23.21 ± 2.10	0.337 ± 0.077
Irinotecan + Micelles	PBS	25	18.60 ± 2.80	0.557 ± 0.103

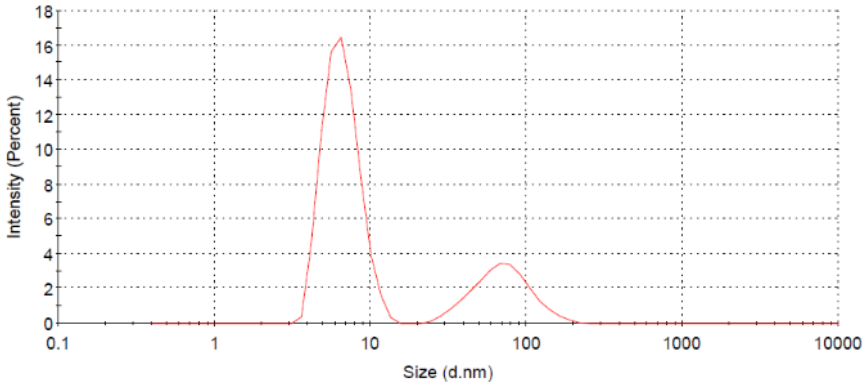

Figure 23 DLS data of empty pluronic F127 at 25 mg/mL in DI water with size populations around 7 nm and 75 nm. Data representative of one independent run.

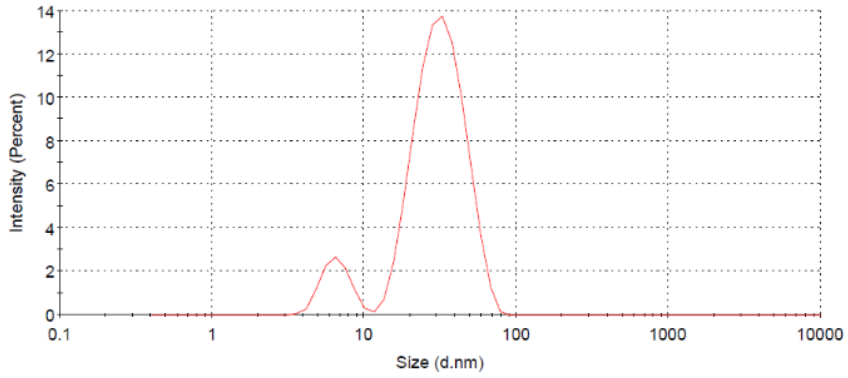

Figure 24 DLS data of empty pluronic F127 at 25 mg/mL in 1x PBS pH 7.4 with size populations around 7 nm and 33 nm. Data representative of one independent run.

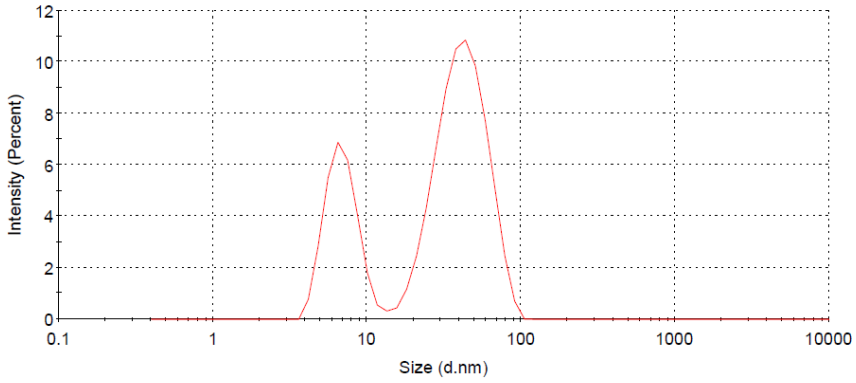

Figure 25 DLS data of irinotecan HCl loaded pluronic F127 at 0.312 mg/mL and 25 mg/mL, respectively, in 1x PBS pH 7.4 with size populations around 7 nm and 43 nm. Data representative of one independent run.

4.4 Cytotoxicity

Mouse melanoma cancer cells (B16F10) were treated with empty Pluronic F127 micelles made in serum free DMEM/High Glucose. The micelles ranged in concentration from 1 mg/mL to 20 mg/mL (0.079-1.59 mM). After 24 hours at 37°C, there was little to no decrease in cell viability. An example of one of the MTS assays is shown in Figure 26.

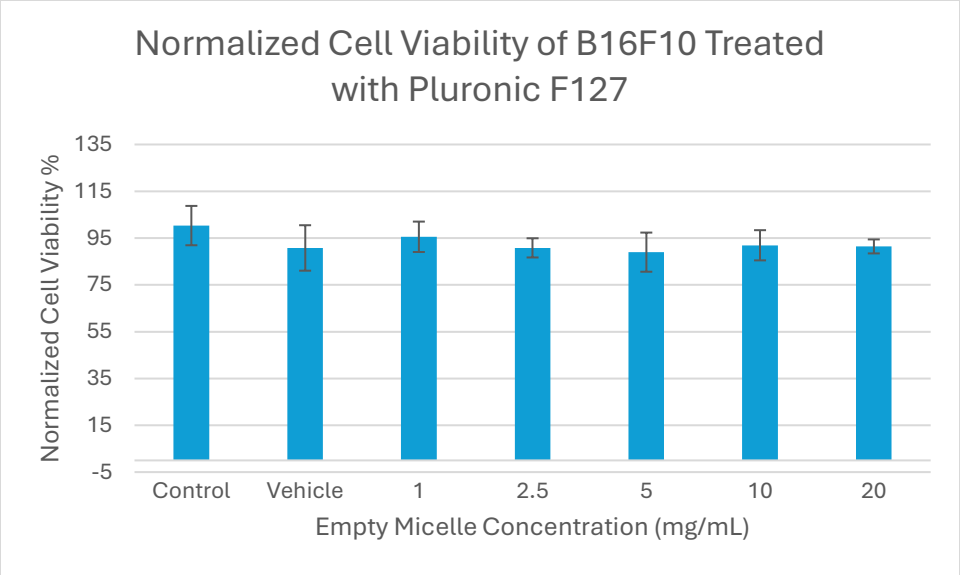

Figure 26 Cell viability of B16F10 cells treated with empty Pluronic F127 (0.1 mg/mL to 20 mg/mL) after 24 hours in serum free DMEM/High Glucose. Data representative of one independent run where the error bars measure the SD of four wells of cells per concentration on one 96-well plate.

Multiple other studies have shown that Pluronic F127 micelles are not cytotoxic to cells, including Meng et al. and Butt et al.[14, 21] Both studies determined the cytotoxicity of Pluronic F127 micelles on brain capillary endothelial cells (BCECs) and mouse lung fibroblast cells (V79s), respectively. Each study used concentrations ranging from 0.1 to 100 µg/mL and 10 to 1000 µg/mL, respectively, and found little to no decrease in cell viability. Both studies used methods other than MTS for determining cell viability. Meng et al. used the cell counting kit-8 (CCK-8) assay; this is a type of colorimetric assay that utilizes 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium (WST-8) salt that reduces to formazan dye in the presence of living cells, and whose absorbance can be directly correlated to cell viability.[23] Butt et al. used the Alamar Blue assay, where living cells convert resazurin to resorufin and the absorbance is directly correlated to cell viability.[24]

Chapter 5: Conclusions and Future Directions

5.1 Conclusions Regarding 1:5 DSPE:TPGS Micelles

The micelle formulation 1:5 DSPE:TPGS had a promising literature background and history of use that suggested it would perform well as a delivery system for PMJ. The micelles had been proven to be an appropriate size that fell within the desired size range of 10-100 nm, as well as to have a very low CMC of 0.0072 mg/mL according to Zhang et al and confirmed by the study here. They also found that the micelles had a high encapsulation efficiency of above 85%. Collectively, these characteristics were optimal for nanoparticles under consideration for drug delivery, and the formulation almost completely delivered on all of these requirements during the research project.

The formulation possessed a desirable CMC of 6 ± 0.002 (n=3) μg/mL (4.06 μM), an EE of around 75% for irinotecan and 85% for PMJ, and a particle size of around 5 nm in DI water, 15 nm in PBS, and around 14 nm when loaded with irinotecan HCl. The corresponding PDIs for all measurements were also below 0.3, indicating that the 1:5 DSPE:TPGS micelles were mostly monodisperse. All these characteristics compared favorably to the data from multiple journal articles that studies this micelle formulation. However, due to the cytotoxic nature of the TPGS, the formulation could no longer be considered for use in the delivery of PMJ to the cells used for this study. The TPGS in the formulation, after a dose curve assay was performed, was seen to kill more than 80% of B16F10 cells at concentrations above 0.01 mg/mL. This necessitated a pivot to a new micelle formulation, Pluronic F127 micelles.

5.2 Conclusions Regarding Pluronic F127 Micelles

Pluronic F127 micelles have been used successfully in the past to deliver entrapped molecules to their designated biological target. As was seen with the 1:5 DSPE:TPGS formulation, the literature on these micelles attributed them with a size within the 10-100 nm target range, as well as with a low CMC of 0.63 mg/mL (0.05 mM).[21] Butt et al. found the encapsulation efficiency (EE) of Pluronic F127 micelles to be around 54 ± 2.6%, and the particle size to be 22 ± 2.02 nm.[21] Meng et al. also found the F127 particles to be 20.03 nm, corroborating Butt et al.'s findings.[14]

This study found that pluronic F127 micelles have a CMC of 0.743 ± 0.151 (n=3) mg/mL (0.063 mM) and an EE of 81.5 ± 4.33% when loaded with irinotecan HCl. Empty micelles made in DI water were 13.2 nm with a PDI of 0.428, and those made in PBS were 23.2 nm with a PDI of 0.337. The key difference between these two solutions is that DI water has an ionic strength of zero, whereas that of PBS is greater than zero. Irinotecan loaded micelles made in PBS were 18.6 nm with a PDI of 0.557. These sizes correspond closely to the particle size noted in the Meng paper. The PDIs for the micelles are all in the realm of somewhat polydisperse, meaning that they all have a small percentage of different particle sizes present in solution. The cytotoxicity of the micelles was also tested against B16F10 mouse melanoma cancer cells and was found to have little to no cytotoxic effects up to 20 mg/mL. This high concentration limit for F127 will allow for maximum flexibility in terms of drug loading amounts and drug copolymer ratios accessible for testing. All in all, the Pluronic F127 micelles are off to an encouraging start in the characterization process.

5.3 Future Directions

There is more work still to be done to fully qualify Pluronic F127 as a delivery system for PMJ. A stability test would need to be done to determine a preliminary shelf life in a controlled environment, as well as a release study to determine how quickly the micelles release entrapped drug. Furthermore, a drug loaded cytotoxicity assay would need to be performed, compared to a free drug assay on the B16F10 cells (ultimately ending with a duplicate treatment on CT26 mouse colon cancer cells under similar experimental conditions). All micelle and drug combination assays will need to be repeated for PMJ to compare results with irinotecan HCl. Finally, after all characterization of the micelle formulation is completed satisfactorily, then animal treatment studies could begin and thus further the journey of PMJ towards clinical trials, and ultimately, use as an approved anticancer treatment for colon cancer

Works Cited

1. Cleveland Clinic medical. (2024, December 19). Cancer staging: How does it work?. Cleveland Clinic. https://my.clevelandclinic.org/health/diagnostics/22607-cancer-stages-grades-system (accessed Jan 17, 2025)
2. American Cancer Society. *Colorectal Cancer Facts & Figures 2023-2025*. Atlanta: American Cancer Society; 2023.
3. Guardiola, M., Buitrago, S., Fernández-Esparrach, G., O'Callaghan, J. M., Romeu, J., Cuatrecasas, M., Córdova, H., González Ballester, M. Á., & Camara, O. Dielectric properties of colon polyps, cancer, and normal mucosa: Ex vivo measurements from 0.5 to 20 GHz. Medical Physics (Lancaster), **2014** *45*(8), 3768-3782. https://doi.org/10.1002/mp.13016
4. Yan S; Wang W; Feng Z; Xue J; Liang W; Wu X; Tan Z; Zhang X; Zhang S; Li X and Zhang C. Immune checkpoint inhibitors in colorectal cancer: limitation and challenges. Front. Immunol, **2024** 15, 1403533. doi: 10.3389/fimmu.2024.1403533
5. Battaglin, F., Naseem, M., Lenz, H. J., & Salem, M. E. (2018). Microsatellite instability in colorectal cancer: overview of its clinical significance and novel perspectives. Clinical advances in hematology & oncology : H&O, 16(11), 735–745.
6. NIH. (2022, August 23). Chemotherapy to treat cancer. NCI. https://www.cancer.gov/about-cancer/treatment/types/chemotherapy (accessed Feb 2, 2025).
7. National Cancer Institute. Irinotecan Hydrochloride. https://www.cancer.gov/about-cancer/treatment/drugs/irinotecanhydrochloride (accessed Jan 29, 2024).

8. American Cancer Society. (2025, January 14). Monoclonal Antibodies and Their Side Effects. https://www.cancer.org/cancer/managing-cancer/treatment-types/immunotherapy/monoclonal-antibodies.html (accessed Apr 4, 2025)
9. Ladin, D. A; Soliman, E; Escobedo, R; et al. Synthesis and evaluation of the novel prostamide, 15-deoxy, Δ12,14-prostamide J2, as a selective antitumor therapeutic. *Molecular Cancer Therapeutics*, **2017**, *16*(5), 838–849. 10.1158/1535-7163.mct-16-0484
10. Zhang, J.; Shen, L.; Li, X.; et al. Nanoformulated Codelivery of Quercetin and Alantolactone Promotes an Antitumor Response through Synergistic Immunogenic Cell Death for Microsatellite-Stable Colorectal Cancer. *ACS Nano,* **2019**, *13*(11):12511-12524. doi: 10.1021/acsnano.9b02875.
11. Kotta, S.; Aldwasari, H. M.; Badr-Eldin, S. M.; Nair, A. B.; YT, K. Progress in Polymeric Micelles for Drug Delivery Applications. *Pharmaceutics*, **2022,** *14,* 1636. https://doi.org/10.3390/pharmaceutics14081636
12. Jin, Y; Wu, Z; Li, C; et al. Optimization of Weight Ratio for DSPE-PEG/TPGS Hybrid Micelles to Improve Drug Retention and Tumor Penetration. *Pharm Res*, **2018,** *35,* 13. https://doi.org/10.1007/s11095-017-2340-y
13. Alexandridis, P; Holzwarth, JF; Hatton, TA. Micellization of Poly(ethylene oxide)-Poly(propylene oxide) Triblock Copolymers in Aqueous Solutions: Thermodynamics of Copolymer Association. *Macromolecules.* **1994**; 27, 2414-2425
14. Meng, X; Liu, J; Yu, X; et al. Pluronic F127 and D- D-α-Tocopheryl Polyethylene Glycol Succinate (TPGS) Mixed Micelles for Targeting Drug Delivery across The Blood Brain Barrier. *Scientific Reports*. **2017**; 7, 2964

15. Malatesta, M. Transmission Electron Microscopy as a Powerful Tool to Investigate the Interaction of Nanoparticles with Subcellular Structures. *Int. J. Mol. Sci*. **2021**, *22*, 12789. https://doi.org/10.3390/ ijms222312789

16. Nano Temper (2022). The biologics researcher's guide to DLS.

17. Arab-Bafrani, Z., Shahbazi-Gahrouei, D., Abbasian, M., & Fesharaki, M. Multiple MTS Assay as the Alternative Method to Determine Survival Fraction of the Irradiated HT-29 Colon Cancer Cells. **Journal of medical signals and sensors**, **2016;** *6*(2), 112–116.

18. Tyler King contributed experimental data to this data set.

19. Evans, J. P.; Winiarski, B. K.; Sutton, P. A.; Jones, R. P.; Ressel, L.; Duckworth, C. A.; Pritchard, D. M.; Lin, Z. X.; Fretwell, V. L.; Tweedle, E. M.; Costello, E.; Goldring, C. E.; Copple, I. M.; Park, B. K.; Palmer, D. H.; Kitteringham, N. R. The Nrf2 inhibitor brusatol is a potent antitumour agent in an orthotopic mouse model of colorectal cancer. *Oncotarget***. 2018** Jun 5;*9*(43):27104-27116. doi: 10.18632/oncotarget.25497. Erratum in: Oncotarget. 2019 Jan 18;10(6):685. doi: 10.18632/oncotarget.26625.. Vicky, Fretwell L [corrected to Fretwell, Vicky L]. PMID: 29930754; PMCID: PMC6007465.

20. Bedo, Zs.; Berecz, E.; Lakatos, I. Colloid Polym. Sci. 1992,270, 799.

21. Butt, A. M.; Amin, M. C. I. M.; Katas, H.; Sarisuta, N.; Witoonsardslip, W.; Benjakul, R. In Vitro Characterization of Pluronic F127 and D-α-Tocopheryl Polyethylene Glycol 1000 Succinate Mixed Micelles as Nanocarriers for Targeted Anticancer-Drug Delivery. *Journal of Nanomaterials,* **2012**, *1*. https://doi.org/10.1155/2012/916573.

22. Gutiérrez-Saucedo, R. A., Gómez-López, J. C., Villanueva-Briseño, A. A., Topete, A., Soltero-Martínez, J. F. A., Mendizábal, E., Jasso-Gastinel, C. F., Taboada, P., & Figueroa-Ochoa, E. B. (2023). Pluronic F127 and P104 Polymeric Micelles as Efficient

Nanocarriers for Loading and Release of Single and Dual Antineoplastic Drugs. Polymers, 15(10), 2249. https://doi.org/10.3390/polym15102249

23. Tocris. Protocol for Cell Counting Kit-8. https://www.tocris.com/resources/protocols/protocol-cell-counting-kit-8. (accessed Apr 11, 2025).
24. ThermoFisher Scientific. AlamarBlue Assays for Cell Viability. https://www.thermofisher.com/us/en/home/life-science/cell-analysis/fluorescence-microplate-assays/microplate-assays-cell-viability/alamarblue-assay-cell-viability.html. (accessed Apr 11, 2025).
25. Andrews, S. S.; Tretton, J. Physical Principles of Circular Dichroism. *Journal of Chemical Education,* **2020;** *97* (12), 4370-4376. DOI: 10.1021/acs.jchemed.0c01061
26. Tranter, G. E. (2017). Protein structure analysis by CD, FTIR, and Raman spectroscopies. Encyclopedia of Spectroscopy and Spectrometry, 740–758. https://doi.org/10.1016/b978-0-12-409547-2.12099-2

Appendix: Structural Determination of 15-deoxy, $D^{12,14}$,-prostaglandin J^2-ethanolamide

NMR Data:

Proton NMR was used to verify product identity. For proton NMR, the instrument used was a Bruker Ascend 400 located at ECU. NMR is a legitimate and established tool for structure determination. The spectrum obtained from one of these verification experiments is depicted in Figure 27 and the spectrum was fully assigned in conjunction with the exception of C12-C13, which were determined by analysis of the H,H J-coupling values.

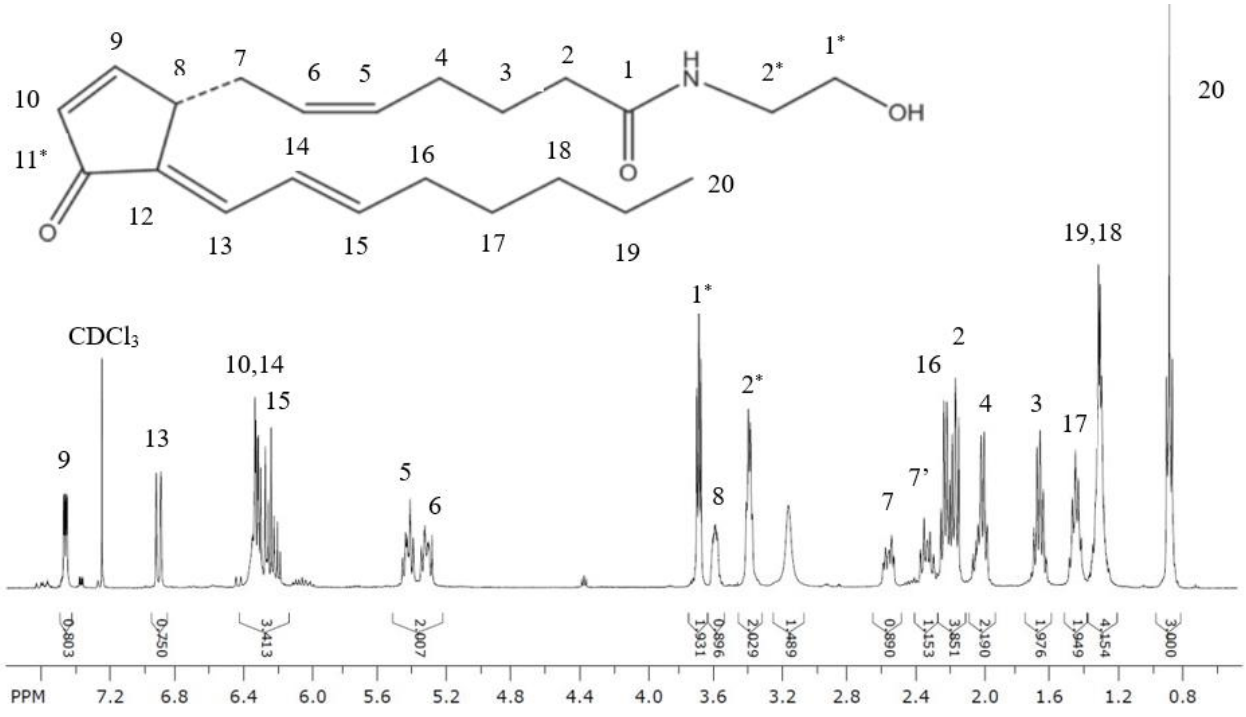

Figure 27 H^1 NMR spectrum for PMJ in $CDCl_3$.

VCD Data:

Vibrational circular dichroism (VCD) was utilized to determine the stereochemistry of PMJ. A 7 mg sample of PMJ was sent to BioTools Inc (Jupiter, FL) for VCD analysis and there it was run as a $CDCl_3$ solution. This technique exposes molecules to left circularly polarized light (LCP) and right circularly polarized light (RCP). Due to the inherent nature of chiral molecules and proteins to be asymmetrical, the molecules will absorb LCP and RCP light differently, and this difference is graphed versus wavelength to ascertain the structural properties of the molecules.[25] VCD is a subtype of CD that utilizes IR light, whereas CD uses light ranging from the UV to the visible region. Using VCD in conjunction with ab initio calculations of the VCD spectra of both enantiomers of a molecule of interest allows for the determination of the absolute configuration of small molecules.[26] The spectrum for the data obtained from VCD is depicted in Figure 28.

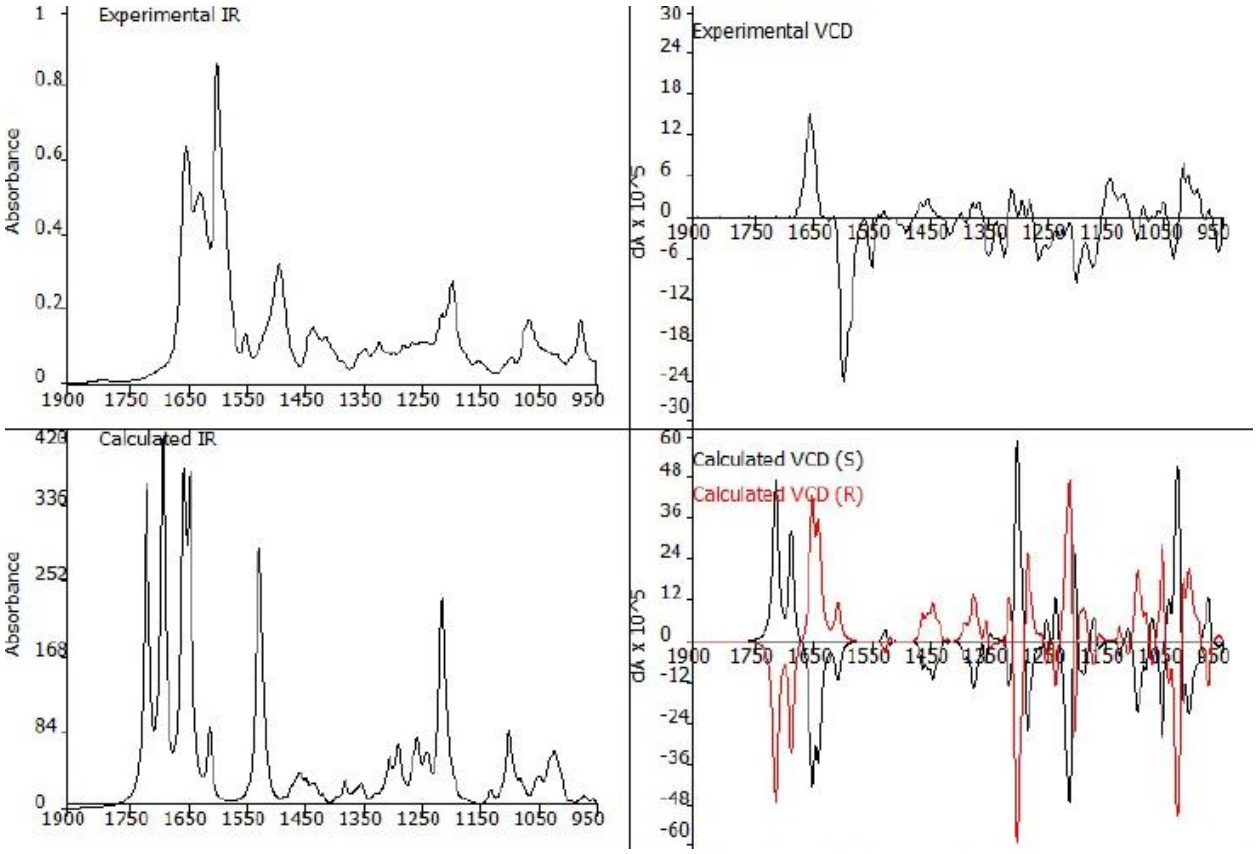

Figure 28 Computer and experimentally generated VCD data for PMJ form BioTools Inc. in Jupiter, Florida.

The absolute configuration was determined to be S at carbon 8, with a confidence level of 99%. The confidence level is a measure of the degree of congruence between the calculated and measured spectrum. It should be noted that the confidence level is not the likelihood that the assignment is correct. Rather, it's a measure of quality or degree of agreement between calculated and measured spectra. With a confidence level of 99% for this molecule, the visual agreement between measured and calculated spectra is very good which gives a high level of confidence in the assignment as S.